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Environmental DNA as an effective tool for detection of imperiled fishes

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Abstract Detection of aquatic species is imperfect, especially if the species is rare and exhibits spatial and temporal variability. Many fish species require a number of sampling trips before detection is positive. And yet, information on species persistence is critical for effective conservation efforts. New forensic genetic techniques, such as environmental DNA (eDNA), have been developed and successfully used to validate the presence of exotic aquatic species in new areas. We compared detection of a federally listed, threatened, migratory fish species the Slackwater darter (*Etheostoma boschungi*); using eDNA to concomitantly collected field collections. Detection probabilities for this species are variable, but consistently low in recent collections. Our results indicated that detection using eDNA was vastly more effective than traditional sampling at confirming the presence of the Slackwater darter. Positive detection at non-breeding sites was half of the detection rate at breeding sites, most likely to the greater area available in non-breeding streams. These data suggest that eDNA is an effective tool for quickly evaluating a relatively large number of sites for the presence of rare aquatic species.

Keywords Environmental DNA · Biodiversity inventories · Percidae · Slackwater darter

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Introduction

Detection of rare species is a persistent problem in conservation biology. When detection probability is low, resources are needed for repeated sampling efforts. Even then, if a species is not detected, questions remain: Was it present but not collected? Was the timing of sampling incorrect for detection? A relatively newly employed technique provides a mechanism for the detection of rare species, even at very low densities: environmental DNA, or eDNA. Use of eDNA allows for species detection without any direct contact. Instead, indicators such as slime, scales, epidermal cells, and feces are left behind by the organism and used for detection. The period of time for persistence of DNA depends largely on the environmental setting. DNA can be directly obtained from environmental samples as a method of presence or absence of a species in a particular habitat. This technique has been used successfully in the Great Lakes system to track the invasion progress of a harmful carp species (Jerde et al. 2011, 2013). Further, this technique has been used and empirically tested in several recent studies (e.g. Darling and Mahon 2011; Goldberg et al. 2011; Thomsen et al. 2012; Moyer et al. 2014).

Known from only a few disjunct locations in Alabama and Tennessee (Fig. 1 inset), the Slackwater darter (*Etheostoma boschungi),* is a rare and sporadically distributed percid fish. Slackwater darters inhabit medium sized streams, and like other members of the subgenus *Ozarka*, migrate upstream to spawn in ephemeral seeps, springs, flooded fields, or flooded woods during the winter months when water levels are high (Boschung 1979). In general, springs are extremely vulnerable to disturbance and are among the most exploited of the freshwater habitats. Due to its limited distributional range and a decline in abundance, the Slackwater darter is listed as federally threatened by the United States Fish and Wildlife Service (USFWS) (Boschung and Mayden 2004). Given the current status, the Slackwater darter is often undetected by traditional sampling methods of seining and dip-netting, and detectability has declined significantly in recent decades (Johnston et al. 2013). Our objective was to compare detection of an imperiled fish species using traditional sampling methodology and eDNA.

Methods

A total of 49 localities were sampled both for water for eDNA analysis and for Slackwater darters. These sites represented historical sites for the species, as well as a stream system not traditionally included in surveys for the species (Limestone Creek), but where one specimen of Slackwater darter was recently collected. Thus, we used Limestone Creek as a model for use of eDNA as a reconnaissance tool. Breeding sites were sampled in February and March during the breeding season, while non-breeding sites were sampled in July and August (Fig. 1) during the non-breeding season (Field Permit: C.E. Johnston, Alabama Department of Conservation and Natural Resources). For specific descriptions of

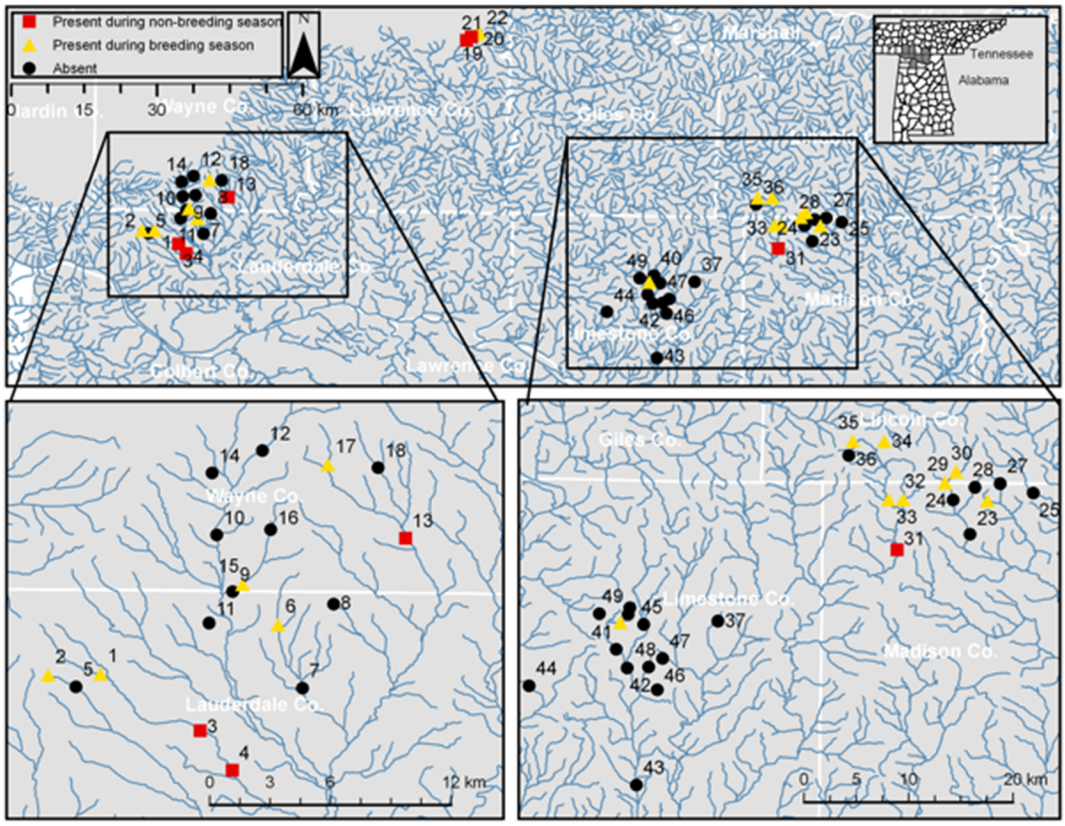

Fig. 1 Non-breeding and breeding sampling sites (n=49) for *Etheostoma boschungi* across the known distributional range. *Red squares* indicate eDNA presence during the non-breeding season. *Yellow triangles* indicate eDNA presence during the breeding season. *Black circles* indicate absences during both the breeding and non-breeding seasons

breeding and non-breeding sites, see Johnston et al. (2013). Environmental DNA collection procedure is similar to Mahon et al. (2010) and Jerde et al. (2011). Specifically, at each collection site, water for eDNA was collected first and then fishes were sampled traditionally by using 10-ft seines and dip-nets for 30 min by kick-seining in compliance with permit guidelines. Quality control measures per Mahon et al. (2010) were taken at each site to avoid contamination. Water was collected in bleached, autoclaved one-liter Nalgene® bottles, and collected at three sites in a downstream to upstream fashion along a 150 m transect at approximately the 0, 75, and 150 m marks. On several different collecting trips, two-liters of surface water were collected at the 0, 75, and 150 m marks at a sample locality to improve coverage of the system. As a control measure, two Nalgene® bottles were filled with deionized water before collection, placed into sterilized coolers, and taken into the field to test for contamination. As an additional control mechanism, water samples were collected outside of the Slackwater darter distribution in a tributary of the Elk River. Temperature, depth, and geographic coordinates were collected at every sample location. Within 24 h of collection, water samples were vacuum-filtered onto 1.5 μm pore size glass fiber filters (see Turner et al. 2014). To ensure equipment control, deionized water was passed through each sterilized filter apparatus prior to each sample filtration. After filtration, sample filters were placed in 50 ml conical tubes and stored at −20 °C until further processing.

DNA was extracted from filters using the PowerWater DNA Isolation kit (MOBio Laboratories Inc., Carlsbad, CA) following manufacture's recommendations, with the exception of the final DNA elution step. Final DNA elution was done using deionized water instead of the provided buffer solution. Extraction blanks and controls were included for all DNA extractions and tested negative in subsequent PCRs. There is no evidence of contamination at any step of sample collection, DNA extraction, and PCR.

Molecular markers were designed for the Slackwater darter using publically available sequence information (GenBank, www.ncbi.nlm.nih.gov). To ensure species specificity we targeted a short fragment of the cytochrome *b* gene of mitochondrial DNA and used the BLAST (Basic Local Alignment Search Tool; Genbank, www.ncbi.nlm.nih.gov). Although no species in the watershed are closely related to the Slackwater darter (*Etheostoma boschungi),* to ensure species specificity, we compared all available sequence data of any potential, through distantly related species and non-target species common to the watershed, (*E. caeruleum, E. corona, E. flabellare, E.nigripinne, E. rufilineatum, E. tennesseense*). We also tested the markers against tissue-derived DNA for a subset of the species listed above. Primers do not amplify species outside the genus *Etheostoma.*

Etheostoma boschungi DNA presence was then assessed using polymerase chain reactions (PCR) for each filtration control, water sample, and cooler control. Twenty-five microliter PCR reactions were performed using 1 μL of template DNA, 14.5 μL of Qiagen Master Mix, 2.5 μL of $MgCL_2$, 2.5 μL dNTPs, 1 μL of each primer (10 μM; L: GTGACTTGAAAAAACCACCG TTC, H: CAACGATCTCCGGTTTACAACAX) under the following thermal cycling conditions: an initial incubation at 94 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min, followed by a final extension at 72 °C for 7 min (Qiagen, Inc., Valencia, CA). Both negative and positive controls were run for each PCR reaction. Multiple PCR reactions were run for each sample to ensure validity of results (Ficetola et al. 2014). PCR results were visualized under UV light on a 1 % agarose gel stained with ethidium bromide. Negative and positive controls were run for each gel. For approximately 10 % of all positive samples, bands were extracted from the agarose gel using the Qiagen QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA) and bi-directionally sequenced by GenWiz, Inc. (New Brunswick, NJ). Sequence data were aligned and also screened using BLASTn (GenBank).

Results

Using traditional sampling methods (seines and dip nets) Slackwater darters were detected at 1 of the 49 sites surveyed (Online Resource 1). This site, (a tributary of Cypress Creek, site 17, Fig. 1) is the only historical locality that has 100 % detectability of Slackwater darter during both the breeding and non-breeding seasons (Johnston et al. 2013). Environmental DNA from water samples was positive for Slackwater darter at this site as well. Further, eDNA methods provided a positive signature of amplification for Slackwater darters at 16/29 of the breeding sites and 7/20 of the non-breeding sites (Fig. 1, Appendix S1), in total: 23/49 sites surveyed.

Detection of eDNA was significantly more effective (McNemar's chi-squared test with continuity correction (McNemar 1947) χ^2=20.0455, *df*=1, p-value = 7.562× 10^{-06}) than traditional sampling. Positive detection of eDNA was sampled at all sites within the Lindsay Creek system, where recent detection of Slackwater darter has been very low (Johnston et al. 2013). Positive eDNA was found at Greenbrier Branch and a seep at the Tennessee/Alabama state line, where Slackwater darter had not been found since the 1970's (Johnston et al. 2013). Although no Slackwater darters were collected, water samples provided DNA signature from the target species for all sites in the Shoal Creek system as well as Chief Creek (Buffalo River drainage). Three sites in the Flint River system were positive for eDNA, while Slackwater darter DNA was only found at one site in Swan Creek.

Limestone Creek was chosen as a model for using eDNA as a reconnaissance tool. Although no specimens were collected, positive eDNA was detected at 5/6 sites sampled in this large watershed (Fig. 1).

Detection of eDNA was also more effective at breeding sites during breeding season (56 %) than at non-breeding sites during non-breeding season (35 %). This may be due to the larger area of non-breeding sites. All sequences from positive samples matched the target species and were aligned and screened with BLASTn (GenBank). No positive signatures were detected from filter controls, cooler controls, or the water samples taken outside of the distribution of Slackwater darter. While false negatives are a possibility, no detection is an inevitable possibility with both traditional and eDNA sampling. Our results demonstrate that eDNA is able to detect species presence when traditional sampling methods fail.

Of the three PCR replicate samples taken at each sampling site along the 150 m transect, 70 % were positive from all three replicates along the 150-m transect (upstream, middle, and downstream), while 30 % of samples sites had a positive DNA Slackwater darter signature from only one or two of the replicates. There does not appear to be a correlation with spatial position of the sample, however.

Discussion

Environmental DNA was vastly more effective at detection of Slackwater darter than traditional methods in the study of Slackwater darters. Higher detection rates using eDNA were accomplished in a short period of time through the use of standard PCR, while traditional sampling can take several years before positive detection is achieved for this species (Johnston et al. 2013). As multiple samples from multiple locations were positive, standard PCR procedures proved sufficient for this scale of study. Specifically, Nathan et al. (2014) demonstrate little difference in detection probability in the mesocosm experiments when using standard PCR, quantitative PCR, and digital droplet PCR. Further, conservation measures for this species require evidence of presence in a timely manner. Detection of Slackwater darter using eDNA was more effective at breeding sites during the breeding season than at non-breeding sites during the non-breeding season. This is not surprising, as the streams used as non-breeding habitat are much larger and have higher discharge than the seepage areas used for spawning (Boschung and Mayden 2004).

Limestone Creek samples illustrate how eDNA can be used to screen an entire watershed for the presence of rare aquatic species. A single specimen of Slackwater darter was collected in this creek system (J. Powell, 5/25/99, Limestone Creek near Toney, AL; 34.916111 N, 86.748611 W), however potential breeding sites in this creek system are unknown. Our samples suggest that the Slackwater darter uses multiple breeding sites, and thus we have narrowed these areas down for future empirical sampling.

The Slackwater darter is a migratory species in steep decline (Mirarchi et al. 2004). Over time, detection ability has decreased, even at breeding sites during the breeding where the fish are concentrated. Johnston et al. (2013) showed a 45 % distributional decline from data collected over a 10-year period. Low rates of detection necessitate multiple sampling trips, often over multiple years and or breeding seasons, which is expensive. Conservation measures often require confirmation of species presence within a short time frame. Traditional sampling with no success can be both exhausting and resource intensive. This technique (eDNA) offers a more cost-effective method for determining species presence for species similar to the Slackwater darter. Additionally, this tool can be useful for quick reconnaissance of potential sites for the targeted organism. Specifically, localities can be screened for further study using traditional sampling and targeted for protection.

To our knowledge the eDNA evidence presented here is the first use of this technique for rare fishes in

the Southeastern United States, but has successfully been used in other vertebrate applications (e.g. Goldberg et al. 2011; Collins et al. 2012; Dejean et al. 2012; Mahon et al. 2013; Takahara et al. 2013; Thomsen et al. 2012). The use of eDNA for detection of rare aquatic species has numerous applications for conservation. This tool can be particularly useful for migratory species, species that are rare, species that are traditionally difficult to sample, or a combination of all of these factors. The use of eDNA offers the ability to protect habitat and target organism, even when specimens are undetectable using usual sampling methods.

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